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Use of electrofocusing for the analysis and purification of turkey erythrocytes β_1-adrenoceptors

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We show that following one cycle of alprenolol affinity chromatography of turkey erythrocyte β_1-adrenoceptors, electrofocusing on polyacrylamide gels in digitonin, followed by electroelution, results in complete receptor purification. The overall yield from the electrofocusing-electroelution step of turkey erythrocyte β-adrenoceptor is 75 ± 3%. In addition, we are able to demonstrate that receptor-binding assays can be performed directly on the polyacrylamide gel, using ^{125}I-cyanopindolol. This method can be employed for minute quantities of receptor which is an advantage when one wishes to characterize rapidly the β-adrenoceptor in its native state from tissues that may be available only in limited amounts. We also report, for comparison, on the behavior of the turkey erythrocyte β_1-adrenoceptor on immobiline polyacrylamide gels and the ability to purify only partially the receptor on these gels.

β-Adrenoceptor; Electrofocusing; Ampholine; Immobiline

1. INTRODUCTION

β-Adrenoceptors have been thoroughly investigated during the past decade [1]. Recently, a number of laboratories have reported the successful purification of β-adrenoceptors from various sources. In all instances reported, more than one cycle of affinity chromatography on an alprenolol affinity column is required in order to achieve over 500–1000-fold purification of the receptor. Benovic et al. [2] pointed out that complete purification beyond affinity chromatography requires a sizing step on HPLC [2]. May et al. [3] have used ion-exchange chromatography in order to achieve complete purification. In order to process small amounts of receptor we have developed an isoelectric focusing step in digitonin, following one cycle of alprenolol affinity chromatography.

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Abbreviations: CYP, cyanopindolol; PEI, polyethylenimine; TEMED, *N,N,N',N'*-tetramethylethylenediamine; PMSF, *p*-methylphenylsulfonyl fluoride; DHA, dihydroalprenolol; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Materials*

Acrylamide and bisacrylamide were obtained from Bio-Rad (USA). Ampholines were a product of Pharmacia (Uppsala) or LKB (Bromma, Sweden). Immobiline was obtained from LKB and SDS from Bio-Rad. l- and d-Propranolol were obtained from ICI (England) while digitonin was purchased (over 99% pure) from Serva (Heidelberg). Unless otherwise indicated, all other chemicals were obtained from Sigma (St. Louis, MO).

2.2. *Alprenolol affinity gel*

Alprenolol-amine and Affi-Gel 10-alprenolol-amine were prepared as described [4].

2.3. *[^{3}H]DHA binding*

Binding of [^{3}H]DHA to soluble β_1-adrenoceptors was conducted as in [4], using PEI-coated GF/B glass filters [5].

2.4. *Preparation of membranes*

Plasma membranes from turkey erythrocytes were purified according to Puchwein et al. [6] and stored at −70°C.

2.5. *Purification of β_1-adrenoceptors*

Turkey erythrocyte membranes (250 mg protein) were

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solubilized with 1% digitonin in buffer containing 10 mM Tris-HCl (pH 7.4), 90 mM NaCl and 0.1 mM *o*-phenanthroline, in a final volume of 100 ml, for 2 h at 4°C. The solubilized material was gently stirred with 7 g alprenololamine Affigel-10 [4], for 2 h at room temperature. The loaded gel was washed with 40 ml of 10 mM Tris-HCl (pH 7.4), 90 mM NaCl, 0.08% digitonin containing 20 μM PMSF, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml bacitracin and 2×10^{-4} M benzamidine, as protease inhibitors. The receptor was eluted overnight in 35 ml of a solution containing 0.1 mM l-alprenolol in 10 mM Tris-HCl (pH 7.4), 250 mM NaCl, 0.2% digitonin, in the presence of protease inhibitors, at 4°C. The eluate was concentrated to 1 ml, and excess of ligand was removed by G-50 chromatography. The receptor was concentrated in an Amicon microconcentrator to 10 pmol receptor/ml, as determined by [^{3}H]DHA binding.

2.6. *Isoelectric focusing of purified receptor*

Analytical polyacrylamide isoelectric focusing was performed on 0.5-mm thin-layer polyacrylamide gels, over the range pH 3.5–9.5, containing 0.08% digitonin. Gels were run for 5 h at 5°C at 2000 V. Samples (20 μl) containing 200 fmol receptor were loaded at each lane. Pharmacia PI markers were run parallel to the receptors.

The receptor was detected on the gel, after focusing by ^{125}I-CYP binding, as follows: Subsequent to focusing, the gel was cut into parallel lanes and each lane immersed in 10 mM Tris-HCl (pH 7.4), 90 mM NaCl buffer for 10 min, in order to equilibrate the gel with the pH of the binding buffer. The strips were then transferred to a solution of 150 pM ^{125}I-CYP, with or without 10 μM propranolol, and incubated at 30°C for 20 min. The gels were then washed 5 times with double-distilled water and dried. Radioactivity remaining in the gels was localized by autoradiography overnight, using X-ray films.

Protein was visualized on the gel by silver nitrate staining according to [7].

2.7. *Analytical electrofocusing in immobilized pH gradient*

This was performed in a pH 5.5–7.5 gradient, according to LKB application note N.324. The gel was coated between two glass plates, one with a 250 × 120 × 0.8 mm U frame. A gel bond PAG film was carefully attached to the glass without the U frame. The two glass plates were clamped on three sides, and the cassette was put on a level surface. The gradient mixture was put on a magnetic stirrer 5 cm above the top of the cassette. The basic light solution contained: 150 μl immobiline, pH 3.6; 118 μl immobiline, pH 6.2; 143.5 μl immobiline, pH 7.0; 142 μl immobiline, pH 8.5; 1.25 ml acrylamide, 29.1% + 0.9% bisacrylamide, and double-distilled water to a final volume of 7.6 ml. The pH of the basic starting solution was measured before the addition of acrylamide and was 7.5. The solution was poured into the reservoir. The acidic, dense solution contained: 228 μl immobiline, pH 4.6; 177 μl immobiline, pH 6.2; 57.5 μl immobiline, pH 7.0; 1.25 ml acrylamide and bisacrylamide; 10% glycerol and double-distilled water were added to a final volume of 7.6 ml. The pH of this solution, as measured before acrylamide addition, was 5.5. The solution was poured into the mixing chamber of the gradient mixer. 25 μl of 10% TEMED and 25 μl of 10% ammonium persulfate were added to each chamber. After filling, the gel mould was put in an oven at 50°C for 1 h, and subsequently the gel was washed for 1 h in double-distilled water to remove free immobiline and catalyst. The gel was dried back to its original weight and placed in an LKB ultrophore for pre-running. NaOH (10 mM) was used as the cathodic solution, while 10 mM glutamic acid was used as the anodic solution. Pre-running conditions were: voltage, 2000 V; current, 10 mA; power, 5 W; temperature, 10°C; time, 45 min. The receptor was applied to sample wells according to the experimental conditions, and the gel was focused overnight under the above-described conditions. The receptor was detected on the gel by ^{125}I-CYP binding, as described above.

2.8. *Preparative electrofocusing*

Preparative electrofocusing was performed on polyacrylamide gels under conditions identical to those employed for analytical electrofocusing, except that 4–6 pmol receptor were loaded in a long lane of 100 × 5 mm. After focusing, the polyacrylamide strip which possessed ^{125}I-CYP binding activity was cut out. The receptor was electroeluted overnight at 4°C with electroelution buffer, containing 32 g glycine and 6 g Tris in 1 l, at pH 8.5. The current used was 10 mA. The percentage of recovered receptor was measured by [^{3}H]DHA binding.

2.9. *SDS-PAGE*

This was performed as described [8], using 7.5–15% polyacrylamide gel.

3. RESULTS AND DISCUSSION

Affinity gel purified receptor was used in most of the experiments. The receptor was focused on polyacrylamide gels over the range pH 3.5–9.5, and detected on the gels after focusing by ^{125}I-CYP binding to the receptor on the gel, as described in section 2. In order to determine whether the binding of ^{125}I-CYP was specific, binding activity was determined in the presence or absence of the stereoisomers of propranolol. From fig.1, it can be seen that ^{125}I-CYP binding to the receptor is specific. It is strongly inhibited by l-propranolol but much less so by d-propranolol.

The specificity of ^{125}I-CYP binding to the receptor in the gel was further verified by using other stereoisomers of both agonists and antagonists. Fig.2 shows that the l-stereoisomers of all the agonists and antagonists tested compete for ^{125}I-CYP binding to the receptor on the gel, whereas the d-isomers were found to be much less effective. These results demonstrate that ^{125}I-CYP can be safely used to monitor β-adrenoceptors directly on a polyacrylamide gel when electrofocusing in digitonin. When the receptor was electroeluted from the ^{125}I-CYP identified area on the gel after focusing, [^{3}H]DHA binding to the eluted receptor indicated that about 70–80% of the loaded receptor was recovered from the gel (table 1).

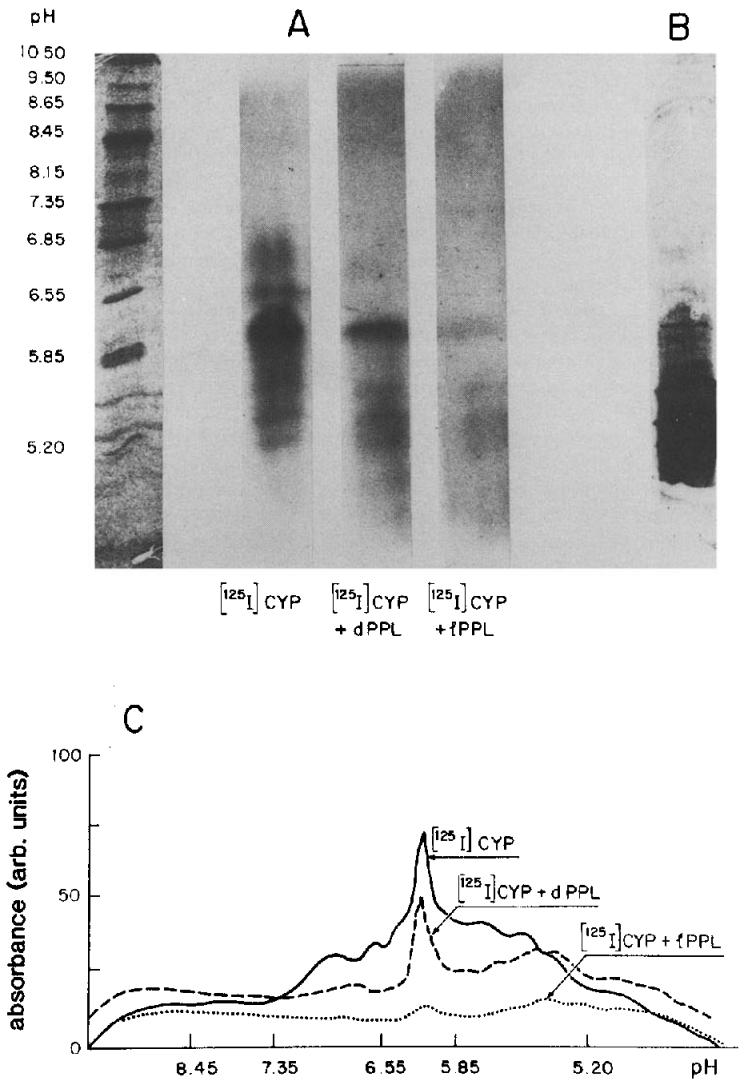

Fig.1. Complete purification of turkey erythrocyte β_1-adrenoceptor on polyacrylamide gel isoelectric focusing. Isoelectric focusing of partially purified receptor. (A) Autoradiography of focused receptor after binding of ^{125}I-CYP in the presence and absence of propranolol stereoisomer. (B) Protein silver staining of receptor after isoelectric focusing. (C) Scanning of the autoradiogram of the above treatment (A).

3.1. *Purity of the affinity-purified and electrofocused receptor*

The affinity-purified receptor is highly contaminated by other proteins, as can be judged from the silver-staining pattern (fig.1B). It should be pointed out that most, if not all, of the contaminating proteins possess an isoelectric pH (p*I*) below that of the β_1-adrenoceptor, the p*I* of which is 6.35. The wide separation between the β_1-adrenoceptor protein and the acidic containing proteins allows, in fact, the complete purification of the receptor by alprenolol affinity chromatography followed by isoelectric focusing. Presumably acidic proteins become attached to the alprenolol column by virtue of its properties as an anion-exchange column, besides the property of being an affinity column for β-adrenoceptors. When the electroeluted receptor is analyzed by SDS electrophoresis and silver staining, it is found to consist mainly of 3 protein bands: P43, P52 and

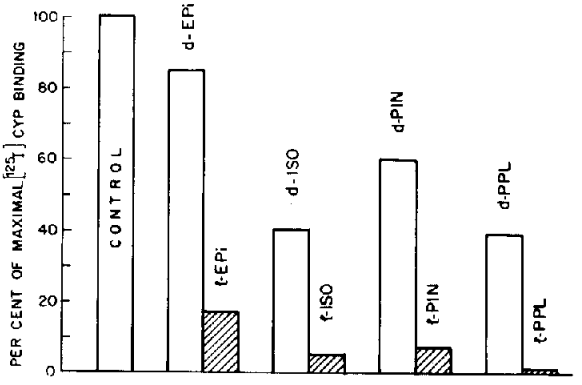

Fig.2. Inhibition of ^{125}I-CYP binding by β-adrenergic ligands on the IEF gel. Binding of ^{125}I-CYP to the gel after isoelectric focusing in the presence of several agonist and antagonist stereoisomers. Results are expressed as percent of binding of ^{125}I-CYP in the absence of other ligand. The experimental protocols used were identical to those in fig.1 but using a variety of ligands. d- and l-epinephrine were used at 100 μM, d- and l-isoproterenol (iso) at 100 μM, l- and d-pindolol (PIN) at 10 μM, d- and l-propranolol (PPL) at 10 μM.

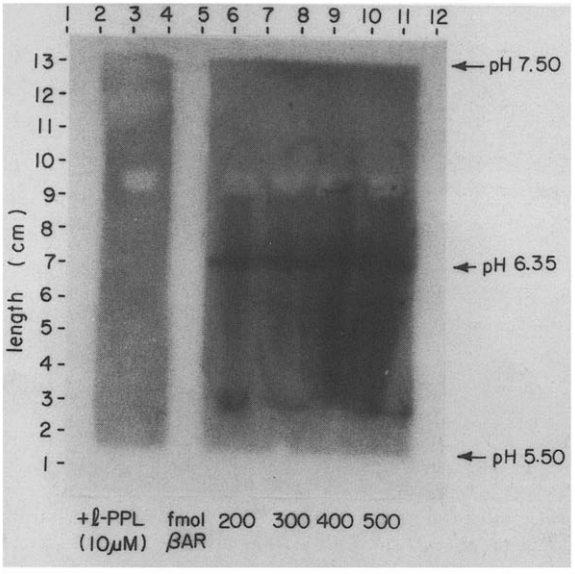

Fig.3. Isoelectric focusing of affinity purified β_1-adrenoceptor on immobilized pH gradient (immobiline). As in polyacrylamide/ampholine gels the major ^{125}I-CYP-binding activity occurs at pH 6.35.

P65 (fig.4). P43 is typical for the turkey erythrocyte β_1-adrenoceptor [9]. We do not as yet know whether the P52 and P65 bands are a genuine receptor species, although Cubero and Malbon [10] have shown that mammalian β_1-adrenoceptors are represented by a subunit of that size. It is feasible that P52 and P65 are more highly glycosylated forms of the receptor and are also present in the avian system.

3.2. *Electrofocusing on immobiline*

Since isoelectric focusing on immobilized pH gradients has many advantages over ampholine gradients [11], we tried to run affinity-purified β_1-adrenoceptors on immobilized gel over the pH gradient 5.5–7.5. For determination of the loading capacity of the polyacrylamide immobilized pH gel, different amounts (200–500 fmol) of partially purified receptor were loaded in different lanes. The receptor was focused at 4°C overnight and detected by binding of ^{125}I-CYP directly to the gel, as described above. Autoradiography of the gel is depicted in fig.3. The receptor is largely focused on a lane at pH 6.35, as observed for the ampholine focused receptor, but there is also a smear along the gel. This smear is negligible when 200–400 fmol receptor are used, although it becomes more significant with quantities of receptor which exceed 500 fmol. The smear may be due to the digitonin and NaCl present in our sample. As the aim of this work is to elute active receptor from the gel after focusing, the sample must be maintained under conditions which will retain its activity; thus the sample could not be dialyzed against double-distilled water, as normally recommended for this method [11].

For preparative immobilized pH electrofocusing, 4 pmol partially purified receptor were loaded on 0.5-mm gels in a long lane of 100 × 0.5 cm, in

Table 1

Yield of electrofocusing and electroelution in the purification of turkey erythrocyte β_1-adrenoceptor

Step	pmol	Overall yield (%)
Membranes (100 mg)	115 ± 10	100
Solubilizate	68 ± 10	59 ± 8.6
Affinity purified (one cycle)	25 ± 6	22 ± 5.0
Electrofocusing[a] and electroelution	20 ± 2	17.4 ± 1.7

[a] Performed in portions of 4–5 pmol each, as described in the text for preparative electrofocusing. Results are for triplicate experiments plus SE

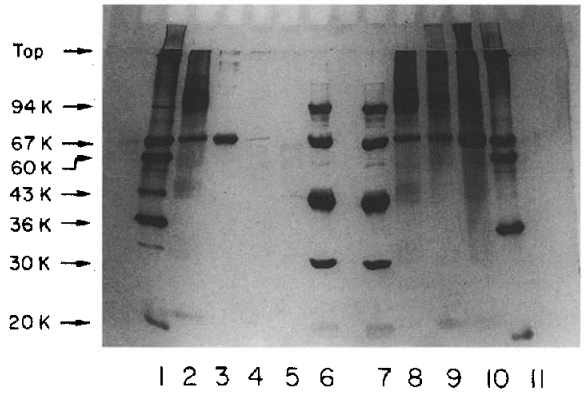

Fig.4. SDS-PAGE of receptor eluted from immobilized pH gradient and ampholine gels. Lanes: 1,11, high-molecular-mass markers; 6,7, low-molecular-mass markers; 2,8, receptor prior to electrofocusing; 3, commercial BSA; 4, receptor eluted from ampholine polyacrylamide gel at the origin; 5, receptor eluted from ampholine polyacrylamide gel at the point of activity; 9, receptor eluted from immobilized gel strip (immobiline) at 0–1 and 3–6 cm from origin; 10, receptor eluted from immobiline gel strip 1.3 cm from origin in the region where the major ^{125}I-CYP-binding activity occurs. K, kDa.

a volume of 340 μl. The receptor was focused overnight and the gel subsequently cut into strips for electroelution. One strip was 1.3 cm from the origin in the anode direction, the other strips being 1.0 and 3.6 cm from the origin. The strips were electroeluted as described above, and the activity of the eluted receptor was determined by [^{3}H]DHA binding. 2.7 pmol (68%) β_1-adrenoceptor were recovered from the strip from the receptor location (1.3 cm from origin, pH 6.35 section), and 0.35 pmol (15%) from the two other strips combined. Since 4 pmol receptor were located on the gel, the overall yield of the receptor after electroelution was about 78%. The extent of purification, using the immobiline method, is significantly less satisfactory as compared to isoelectric focusing using the ampholine method (fig.4). From fig.4, it is clear that the degree of receptor purification is high (lane 5) from the ampholine polyacrylamide gel whereas it is low in immobiline pH gels. There is no significant purification of the receptor eluted from immobiline gels (lane 10) as compared to the receptor prior to electrofocusing (lane 2). On the other hand, there is a high degree of purification of the receptor eluted from the ampholine gel (lane 5).

3.3. *Significance*

The ability to purify extensively β_1-adrenoceptors by electrofocusing on acrylamide gel, in the presence of digitonin, followed by electroelution, may open up new possibilities in the analysis of these receptors and potentially of others. After an affinity chromatography step, the receptor can be easily purified further with high yield in quantities of 100 fmol–5 pmol, using conventional flatbed electrofocusing in digitonin followed by electroelution. The high efficiency of electrofocusing/electroelution, combined with the ability to analyze the β-adrenergic receptor on the gel using ^{125}I-CYP, allows characterization of these receptors.

We have tried to electrofocus digitonin-solubilized receptor, without prior alprenolol affinity chromatography, but have found that the receptor ^{125}I-CYP-binding activity remains at the origin of application, irrespective of its location on the gel (not shown). The method described here enables one to analyse small amounts of receptor and may be adapted to other receptors, especially if they are available in small quantities.

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